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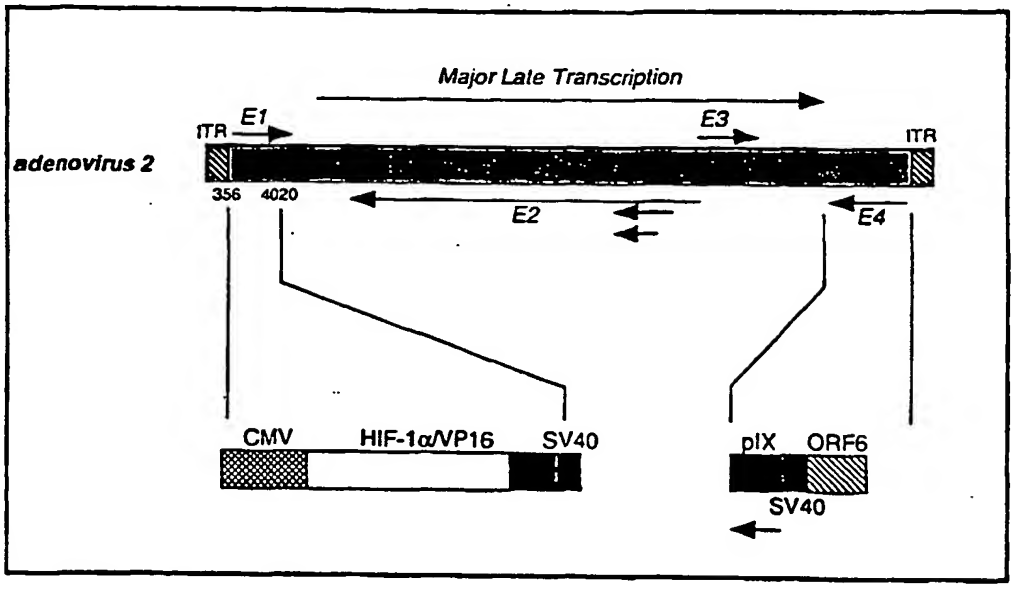


(57) Abstract

The present invention provides recombinant nucleic acid molecules encoding a chimeric transactivator protein including a DNA binding domain of a DNA binding protein and a protein domain capable of transcriptional activation. The present invention also provides recombinant viral and non-viral vectors that are able to infect and/or transfect and sustain expression of a biologically active chimeric transactivor proteins in mammalian cells. Also provided are host cell lines and non-human transgenic animals capable of expressing biologically active chimeric transactivator proteins. In another aspect, compositions and methods for treating or preventing ischemic damage associated with hypoxia–related disorders are provided.

COMPOSITIONS AND METHODS FOR INDUCING GENE EXPRESSION

BACKGROUND OF THE INVENTION

Ischemic heart disease occurs when the heart muscle does not receive an adequate blood supply and is thus deprived of necessary levels of oxygen and nutrients. Ischemia is commonly a result of atherosclerosis which causes blockages in the coronary arteries that provide blood flow to the heart muscle.

Ischemic heart disease can result in certain adaptive responses within the heart which are likely to be beneficial. Among these responses are: 1) increased expression of angiogenic growth factors and their receptors, leading to the formation of collateral circulation around blocked coronary arteries; 2) increased expression of glycolytic enzymes as a means to activate a metabolic pathway which does not require O_2; and 3) expression of heat shock proteins which can protect the ischemic tissue from death.

At least some of these responses appear to be regulated by a complex oxygen sensing mechanism which eventually leads to the activation of transcription factors which control the expression of critical genes involved in this adaptation. Because this altered gene expression occurs only in response to hypoxia, which usually only occurs when a strain such as exercise is placed upon the diseased heart, cardiac patients do not usually receive much benefit from this endogenous compensatory mechanism. As a result, a number of conventional therapies attempt to supplement the natural therapeutic responses of the heart to ischemia.

For example, such treatments include pharmacological therapies, coronary artery bypass surgery and percutaneous revascularization using techniques such as balloon angioplasty. Standard pharmacological therapy is predicated on strategies that involve either increasing blood supply to the heart

the use of adenovirus-mediated gene transfer for regulating function in cardiac vascular smooth muscle.

Accordingly, there exists a need in the art for compositions and methods for inducing the expression of beneficial hypoxia-inducible genes in ischemia-associated cells. Additionally, there exists a need for new vector compositions that allow efficient expression of a range of potentially beneficial genes that are activated by the sustained direct expression of a biologically active mammalian transcription factor. The present invention satisfies these needs and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides recombinant nucleic acid molecules encoding a chimeric transactivator comprising a DNA binding domain of a DNA binding protein wherein the DNA binding protein is a mammalian hypoxia-inducible factor protein, and a functional transcriptional activator domain of a transcriptional activator protein.

Accordingly, in making the invention, we sought to exploit the adaptive response to hypoxia as an alternative approach for the treatment of ischemia associated with vascular disease. We considered that administration of a modified HIF-1α transcription factor via gene therapy might induce expression of a panel of potentially beneficial genes and ultimately lead to the neovascularization of ischemic tissues. We have created a constitutively active form of HIF-1α consisting of the DNA-binding and dimerization domains from HIF-1α and the transactivation domain from herpes simplex virus VP16 protein. Among the possible target genes for this modified transcription factor is VEGF, an endothelial cell-specific mitogen and potent stimulator of angiogenesis.

In vitro analyses of an HIF-1α/VP16 hybrid transcription factor of

In another embodiment, the present invention provides a fusion protein comprising a DNA binding domain of a DNA binding protein wherein the DNA binding protein is the mammalian hypoxia-inducible factor 1 α (HIF-1α) protein at the amino terminus, and a functional transcriptional activator domain of a transcriptional activator protein, wherein said transcriptional activator protein is HSV VP16 at the carboxy terminus.

The present invention further provides a non-human transgenic mammal expressing recombinant DNA encoding a chimeric transactivator comprising a DNA binding domain of a DNA binding protein wherein said DNA binding protein is the mammalian hypoxia-inducible factor 1 α (HIF-1α) protein, and a functional transcriptional activator domain of a transcriptional activator protein, wherein said transcriptional activator protein is HSV VP16.

In yet another embodiment, the present invention provides a method for increasing expression of hypoxia-inducible genes.

In still yet another embodiment, the present invention provides a method for providing sustained expression of biologically active HIF-1α under normoxic conditions.

The present invention also provides a method for treating/preventing/modulating hypoxia-associated tissue damage in a subject.

The present invention further provides a method for providing biologically active chimeric human-viral transactivator protein to the cells of an individual comprising introducing into the cells of an individual an amount of pcDNA3/HIF/VP16/RI or pcDNA3/HIF/VP16/Afl2 effective to transfect and sustain expression of biologically active chimeric human-viral transactivator protein in the cells of the individual.

Other features and advantages of the present invention will be apparent from the following detailed description as well as from the claims.

DETAILED DESCRIPTION OF THE INVENTION

Hypoxia (a state in which O_2 demand exceeds supply) is a powerful modulator of gene expression. The physiologic response to hypoxia involves enhanced erythropoiesis (Jelkman, *Physiol. Rev.* 72:449-489 (1992)), neovascularization in ischemic tissues (White et al., *Circ. Res.* 71:1490-1500 (1992)) and a switch to glycolysis-based metabolism (Wolfe et al., *Eur. J. Biochem.* 135:405-412 (1983)). These adaptive responses either increase O_2 delivery or activate alternate metabolic pathways that do not require O_2. The gene products involved in these processes, include, for example: (i) EPO, encoding erythropoietin, the primary regulator of erythropoiesis and thus a major determinant of blood O_2-carrying capacity (Jiang et al., *J. Biol. Chem.* 271(30):17771-78 (1996); (ii) VEGF, encoding vascular endothelial growth factor, the primary regulator of angiogenesis and thus a major determinant of tissue perfusion (Levy et al., *J. Biol. Chem.* 270:13333 (1995); Liu et al., *Circ. Res.* 77:638 (1995); Forsythe et al., *Mol. Cell. Biol.* 16:4604 (1996)); (iii) ALDA, ENO1, LDHA, PFKL, and PGK1, encoding the glycolytic enzymes aldolase A, enolase 1, lactate dehydrogenase A, phosphofructokinase L, and phosphoglycerate kinase 1, respectively, which provide a metabolic pathway for ATP generation in the absence of O_2 (Firth et al., *Proc. Natl. Acad. Sci., USA* 91:6496 (1994); Firth et al., *J. Biol. Chem.* 270:21021 (1995); Semenza et al., *J. Biol. Chem.* 269:23757 (1994)); (iv) HO1 and iNOS, encoding heme oxygenase 1 and inducible nitric oxide synthase, which are responsible for the synthesis of the vasoactive molecules carbon monoxide and nitric oxide, respectively (Lee et al., *J. Biol. Chem.* 272:5375; Melillo et al. *J. Exp. Med.* 182:1683 (1995)).

An important mediator of these responses is the interaction of a transcriptional complex comprising a DNA binding, hypoxia inducible factor

this response have been localized to the C-terminus of the protein and overlap the transactivation domain. For example, Jiang et al., *J. Biol. Chem.* 271(30):17771-78 (1996) showed that HIF-1α truncated at amino acid 390 lost transactivation activity but retained the ability to bind DNA and showed high levels of protein under both normoxic and hypoxic conditions. This result suggested that the transactivation domain as well as the region conferring instability with normoxia reside in the C-terminal half of the protein. Pugh et al., *J. Biol. Chem.* 272(17):11205-14 (1997) have further localized the regions involved to two areas, amino acids 549-582 and 775-826.

In one embodiment, this invention provides nucleic acid molecules encoding biologically active chimeric transactivator proteins comprising a domain of the HIF-1α protein sufficient for DNA binding and dimerization with HIF-1β (ARNT) and a protein domain capable of transcriptional activation.

In another embodiment, a related DNA binding, hypoxia inducible factor protein is EPAS1. EPAS1 is a PAS domain transcription factor termed endothelial PAS-1. Tian et al., *Genes Dev.* 11:72 (1997). EPAS1 shares 48% identity with HIF-1α and lesser similarity with other members of bHLH/PAS domain family of transcription factors (EPAS1 human sequence GenBank Acc. No. U81984; mouse sequenc GenBank Acc. No. U81983). Like HIF-1α, EPAS1 binds to and activates transcription from a DNA element originally isolated from the EPO gene and containing the HRE core sequence. EPAS1 also forms a heterodimeric complex with ARNT prior to transcriptional activation of target genes.

Human and murine EPAS1 share extensive primary amino acid sequence identity with HIF-1α (48%). Sequence conservation between the two proteins is highest in the bHLH (85%), PAS-A (68%), and PAS-B (73%)

protein. As will be recognized by the skilled artisan, the adaptive response to hypoxia is likely to have been highly conserved throughout evolution. Accordingly, hypoxia inducible factor proteins would be expected to occur in a wide variety of species including non-mammalian vertebrates and non-vertebrates such as insects. See, for example, Bacon et al., *Biochem. Biophys. Res. Comm.*, 249:811-816 (1998), which reports the functional similarity between the Sima basic-helix-loop-helix PAS protein from *Drosophila* and the mammalian HIF-1α protein.

Nucleic acid and amino acid sequences for non-mammalian hypoxia inducible factor proteins may be obtained by the skilled artisan by a variety of techniques, for example by cross-hybridization or amplification using all or a portion of the sequences referred to herein. Once the sequence encoding a candidate hypoxia inducible factor protein has been determined, the localization of portions of the protein sufficient to bind to HREs and dimerize with HIF-1β may be determined using, e.g., the same types of techniques used to determine the location of those domains within the human HIF-1α protein. Relevant domains of non-mammalian hypoxia inducible factor proteins useful in the compositions and methods of this invention may also be produced synthetically or by site-directed manipulations of the DNA encoding known mammalian hypoxia inducible factor proteins. It is also expected that the sequence motifs in common among various mammalian and non-mammalian hypoxia inducible factor proteins will suggest consensus sequences that, while perhaps not occurring naturally in any species, would nevertheless produce domains useful in the methods and compositions of this invention. All that is required in order to substitute such non-mammalian hypoxia inducible factor protein domains for the human HIF-1α protein domains exemplified herein is that they be able to bind to HREs and dimerize with HIF-1β (ARNT).

modify the HIF-1α subunit such that it no longer is destabilized by normoxic conditions and would therefore be more potent, particularly when the patient being treated is not actually ischemic.

To stabilize the hypoxia inducible factor protein under normoxic conditions and to provide strong, constitutive transcriptional activation, a hybrid/chimeric fusion protein consisting of the DNA-binding and dimerization domains from HIF-1α and the transactivation domain from Herpes Simplex Virus (HSV) VP16 protein was constructed. Administration of this hybrid/chimera to the cells of a subject via gene therapy will theoretically induce the expression of genes normally up-regulated in response to hypoxia (i.e., VEGF and the like).

Alternative biologically active chimeric transactivater proteins, such as a protein comprising the DNA binding and dimerization doman from HIF-1α and the transactivation domain from the human NFκB protein, are expected to produce similar results.

"Hypoxia" means the state in which O_2 demand exceeds supply.

"Hypoxia-inducible genes" means genes containing one or more hypoxia responsive elements (HREs; binding sites) within sequences mediating transcriptional activation in hypoxic cells.

Hypoxia inducible factor means a DNA binding protein/transcription factor the expression of which is upregulated under hypoxic conditions, that recognizes and binds to a hypoxia responsive element core sequence within a gene and thereby activates such gene.

Hypoxia-associated disorders include, for example, ischemic heart disease, peripheral vascular disease, ischemic disease of the limb, and the like.

The term "nucleic acids" (also referred to as polynucleotides) encompasses RNA as well as single and double-stranded DNA, cDNA and

like.

In general, nucleic acid manipulations according to the present invention use methods that are well known in the art, as disclosed in, for example, Sambrook et al., *Molecular Cloning, A Laboratory Manual 2d Ed.* (Cold Spring Harbor, NY, 1989), or Ausubel et al., *Current Protocols in Molecular Biology* (Greene Assoc., Wiley Interscience, NY, NY, 1992).

This invention also encompasses nucleic acids which differ from the nucleic acids encoding a human HIF-1α, EPAS1, or HLF, but which have the same phenotype, i.e., encode substantially the same amino acid sequence, respectively. Phenotypically similar nucleic acids are also referred to as "functionally equivalent nucleic acids". As used herein, the phrase "functionally equivalent nucleic acids" encompasses nucleic acids characterized by slight and non-consequential sequence variations that will function in substantially the same manner to produce the same or substantially the same protein product(s) as the nucleic acids disclosed herein. In particular, functionally equivalent nucleic acids encode proteins that are the same as those disclosed herein or that have conservative amino acid variations. For example, conservative variations include substitution of a non-polar residue with another non-polar residue, or substitution of a charged residue with a similarly charged residue. These variations include those recognized by skilled artisans as those that do not substantially alter the tertiary structure of the protein.

A structural gene is that portion of a gene comprising a DNA segment encoding a protein, polypeptide or a portion thereof, and excluding the 5' sequence which drives the initiation of transcription. The structural gene may be one which is normally found in the cell or one which is not normally found in the cellular location wherein it is introduced, in which case it is termed a heterologous gene. A heterologous gene may be derived in whole or in part

Ma and Ptashne, *Cell* 51:113-119 (1987); Courey and Tjian (1988); Mermod et al., *Cell* 58:741-753 (1989)).

One of the most efficient activator domains known is contained in the carboxyl-terminal 100 amino acids of the Herpes Simplex Virus (HSV) virion protein 16 (VP16; Sadowski et al., *Nature* 335:563-564 (1988); Triezenberg et al., *Genes & Dev.* 2:718-729 (1988)). VP16, also known as Vmw65 or alpha -gene trans-inducing factor, is a structural protein of HSV which activates transcription of the immediate early promoters of the virus, including those for ICPO and ICP4 (Campbell et al., *J. Mol. Biol.* 180:1-19 (1984); Kristie and Roizman, *Proc. Natl. Acad. Sci., USA* 81:4065-4069 (1984); Pellet et al., *Proc. Natl. Acad. Sci., USA* 82:5870-5874 (1985)). Although VP16 specifically activates promoters containing the so called TAATGARAT element, the specificity is endowed by a cellular DNA binding protein(s) which is complexed with the amino terminal domains(s) of VP16 (McKnight et al., *Proc. Natl. Acad. Sci., USA* 84:7061-7065 (1987); Preston et al., *Cell* 52:425-434 (1988)).

The present invention provides novel hybrid/chimeric transactivating proteins comprising a functional portion of a DNA binding protein and a functional portion of a transcriptional activator protein. The hybrid/chimeric transactivating proteins of the invention offer a variety of advantages, including the specific activation of expression of hypoxia-inducible genes containing hypoxia responsive elements (HREs), thereby achieving exceptionally high levels of gene expression. Invention hybrid/chimeric transactivating proteins are capable of functioning in vertebrate cells and may include naturally occurring transcriptional transactivating proteins or domains of proteins from eukaryotic cells including vertebrate cells, viral transactivating proteins or any synthetic amino acid sequence that is able to stimulate transcription from a

an inherently flexible process in which there is little, if any, requirement for specific structures or stereospecific protein contacts. It also reviews the variety of different molecules that can function as transcriptional activators, including short peptide motifs (as small as eight amino acids), simple amphipathic helices and even mutagenized domains of proteins unrelated to transcriptional activation.

According to the invention, DNA sequences encoding the DNA binding protein and the transactivating protein are combined so as to preserve the respective binding and transactivating properties of each. In various embodiments of the invention, the DNA encoding the transactivating protein, or a portion thereof capable of activating transcription, may be inserted into DNA at a locus which does not completely disrupt the function of said DNA binding protein. Regions not required for function of DNA binding proteins or transcriptional transactivating proteins may be identified by any method known in the art, including analysis of mapped mutations as well as identification of regions lacking mapped mutations, which are presumably less sensitive to mutation than other, more functionally relevant portions of the molecule. The appropriate recombinant constructs may be produced using standard techniques in molecular biology, including those set forth in Maniatis (Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory (1989)).

The recombinant DNA construct encoding the chimeric transactivator protein may be placed under the control of (i.e., operatively linked to) a suitable promoter and/or other expression control sequence. It may be desirable for the transactivator protein to be placed under the control of a constitutively active promoter sequence, although said transactivator protein may also be placed under the control of an inducible promoter, such as the

(Readhead et al.,*Cell* 48:703-712 (1987)); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, *Nature* 314:283-286 (1985)), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., *Science* 234:1372-1378 (1986)). Of particular interest is the α-myosin heavy chain gene (Subramaniam, et al., *J. Biol. Chem.* 266:24613-24620, (1991)) and the myosin light chain-2 promoter (Henderson et al., *J. Biol. Chem.* 264:18142-18148 (1989) and Ruoqian-Shen et al., *Mol. Cell. Biol.* 11:1676-1685 (1991), both of which are active in cardiac muscle.

In one preferred specific embodiment of the invention, the chimeric transactivator protein is encoded by pcDNA3/HIF/VP16/Afl2, constructed according to methods set forth in Example 1 and Figure 1. In another preferred specific embodiment of the invention, the chimeric transactivator protein is encoded by pcDNA3/HIF/VP16/RI, which is identical to pcDNA3/HIF/VP16/Afl2 except that the VP16 segment is inserted after codon 530 of the HIF-1α coding region.

According to the invention, the hybrid/chimeric transactivator proteins of the invention may be utilized to specifically regulate the expression of genes containing hypoxia responsive elements (HREs). These HREs correspond to a nucleic acid sequence recognized and bound by the DNA binding protein used as the backbone of the chimeric transactivator protein.

In general, the chimeric transactivator proteins of the invention may be used to selectively control the expression of genes of interest. For example, and not by way of limitation, the chimeric transactivator proteins of the invention may be placed under control of a constitutive promoter and may be used to constitutively increase the expression of a gene of interest associated with hypoxia responsive elements (HREs), for example, when it is desirable to produce a particular gene product in quantity in a cell culture or in a transgenic

vector containing, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Other means are well known and available in the art.

The skilled artisan will recognize that when expression from the vector is desired, the polynucleotides/transgenes are operatively linked to expression control sequences. Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression. Similarly, alternative codons, encoding the same amino acid, can be substituted for coding sequences of the human HIF-1α, EPAS1 or HLF polypeptide in order to enhance transcription (e.g., the codon preference of the host cell can be adopted, the presence of G-C rich domains can be reduced, and the like).

Preparations of invention polynucleotides encoding human HIF-1α,

Ringold, *Nature* 337:387 (1989)). Intravenous injection of cationic lipid-plasmid complexes into mice has been shown to result in expression of the DNA in lung (Brigham et al., *Am. J. Med. Sci.* 298:278 (1989)). See also, Osaka et al., *J. Pharm. Sci.* 85(6):612-618 (1996); San et al., *Human Gene Therapy* 4:781-788 (1993); Senior et al., *Biochemica et Biophysica Acta* 1070:173-179 (1991); Kabanov and Kabanov, *Bioconjugate Chem.* 6:7-20 (1995); Remy et al., *Bioconjugate Chem.* 5:647-654 (1994); Behr, J-P., *Bioconjugate Chem.* 5:382-389 (1994); Behr et al., *Proc. Natl. Acad. Sci., USA* 86:6982-6986 (1989); and Wyman et al., *Biochem.* 36:3008-3017 (1997).

Cationic lipids are known to those of ordinary skill in the art. Representative cationic lipids include those disclosed, for example, in U.S. Pat. No. 5,283,185; and PCT/US95/16174 (WO 96/18372), the disclosures of which are incorporated herein by reference. In a preferred embodiment, the cationic lipid is N^4-spermine cholesterol carbamate (GL-67) disclosed in WO 96/18372.

Adenovirus - Adenovirus-based vectors for the delivery of transgenes are well known in the art and may be obtained commercially or constructed by standard molecular biological methods. Recombinant adenoviral vectors containing exogenous genes for transfer are, generally, derived from adenovirus type 2 (Ad2) and adenovirus type 5 (Ad5). They may also be derived from other non-oncogenic serotypes. See, for example, Horowitz, "Adenoviridae and their Replication" in *VIROLOGY*, 2d ed., Fields et al. Eds., Raven Press Ltd., New York, 1990, incorporated herein by reference.

The adenoviral vectors of the present invention are incapable of replicating, have minimal viral gene expression and are capable of expressing a transgene in target cells. Adenoviral vectors are generally rendered replication-defective by deletion of the E1 region genes. The replication-defective vectors

such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of a polynucleotide to a promoter refers to the physical and functional relationship between the polynucleotide and the promoter such that transcription of DNA is initiated from the promoter by an RNA polymerase that specifically recognizes and binds to the promoter, and wherein the promoter directs the transcription of RNA from the polynucleotide.

Promoter regions include specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. Additionally, promoter regions include sequences that modulate the recognition, binding and transcription initiation activity of RNA polymerase. Such sequences may be *cis* acting or may be responsive to *trans* acting factors. Depending upon the nature of the regulation, promoters may be constitutive or regulated. Examples of promoters are SP6, T4, T7, SV40 early promoter, cytomegalovirus (CMV) promoter, mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MMLV) promoter, phosphoglycerate kinase (PGK) promoter, and the like. Alternatively, the promoter may be an endogenous adenovirus promoter, for example the E1a promoter or the Ad2 major late promoter (MLP). Similarly, those of ordinary skill in the art can construct adenoviral vectors utilizing endogenous or heterologous poly A addition signals.

As used herein "promoter" refers to the nucleotide sequences at the 5' end of a structural gene which direct the initiation of transcripiton. Promoter sequences are necessary, but not always sufficient, to drive the expression of a downstream gene. In general, eukaryotic promoters include a characteristic DNA sequence homologous to the consensus 5' TATA box about 10-30 bp 5' to the transcription start site (CAP site). Another promoter component, the CAAT

expressing a chimeric transactivator protein according to this invention into target cells prior to implantation *in vivo* may provide additional advantages to cellular therapy methods in at least two ways. First, the cells may serve as a transport vehicle for the expression construct, resulting in site-directed delivery of the chimeric transactivator protein in any region of the body in which the cells are transplanted. Second, the expression of a chimeric transactivator protein in the implanted cells may aid their survival after implantation, either by allowing them to more easily adapt to any hypoxic conditions which may be present after implant, and/or by stimulating blood vessel development in the region of implantation.

Use of invention vectors to deliver a polynucleotide/transgene to a cell *in vivo* is useful for the treatment of various disorders, for example, in the case of hypoxia-associated disorders such as ischemic heart disease, to a cell in which HIF-1α is absent, insufficient or nonfunctional. Thus, in further embodiments, this invention provides methods for increasing the expression of hypoxia-inducible genes in target cells of a subject in which such increased expression is desired by administering an effective amount of a composition comprising nucleic acid molecule encoding a biologically active chimeric transactivator protein according to this invention in form suitable for expression (e.g., operatively linked to expression control sequences). An "effective amount" refers to an amount which results in expression of biologically active chimeric transactivator protein at a level and for a period of time sufficient to alleviate one or more of the symptoms associated with a hypoxia-associated disorder. Such methods are useful to increase or sustain the expression of HIF-1α and hypoxia-inducible genes in tissues under hypoxic and normoxic conditions.

In related embodiments, the invention provides methods for treating,

polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eukaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al., *Molecular Cloning, A Laboratory Manual 2d Ed.* (Cold Spring Harbor, NY, 1989), or Ausubel et al., *Current Protocols in Molecular Biology* (Greene Assoc., Wiley Interscience, NY, NY, 1992). Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the invention hybrid/chimeric transactivator (fusion) polypeptide.

This invention provides a transformed host cell that recombinantly expresses the invention hybrid/chimeric transactivator (fusion) polypeptides. Invention host cells have been transformed with recombinant nucleic acid molecules encoding chimeric transactivators comprising a DNA binding domain of a mammalian or non-mammalian hypoxia-inducible factor protein and a functional transcriptional activator domain of a transcriptional activator protein. An example is a mammalian cell comprising a plasmid adapted for expression in a mammalian cell. The plasmid contains a polynucleotide encoding a DNA binding domain of a mammalian or non-mammalian hypoxia-inducible factor protein and a functional transcriptional activator domain of a

useful in the treatment of hypoxia-associated disorders.

Transfer of the polynucleotide/transgene to the target or host cells by invention vectors can be evaluated by measuring the level of the polynucleotide/transgene product in the target or host cell. The level of polynucleotide/transgene product in the target or host cell directly correlates with the efficiency of transfer of the polynucleotide/transgene by invention vectors.

Expression of the polynucleotide/transgene can be monitored by a variety of methods known in the art including, *inter alia,* immunological, histochemical and activity assays. Immunological procedures useful for *in vitro* detection of the hybrid/chimeric transactivator (fusion) polypeptide in a sample include immunoassays that employ a detectable antibody. Such immunoassays include, for example, ELISA, Pandex microfluorimetric assay, agglutination assays, flow cytometry, serum diagnostic assays and immunohistochemical staining procedures which are well known in the art. An antibody can be made detectable by various means well known in the art. For example, a detectable marker can be directly or indirectly attached to the antibody. Useful markers include, for example, radionuclides, enzymes, fluorogens, chromogens and chemiluminescent labels.

For *in vivo* imaging methods, a detectable antibody can be administered to a subject, tissue or cell and the binding of the antibody to the polynucleotide/transgene product can be detected by imaging techniques well known in the art. Suitable imaging agents are known and include, for example, gamma-emitting radionuclides such as ^{111}In, ^{99m}Tc, ^{51}Cr and the like, as well as paramagnetic metal ions, which are described in U.S. Patent No. 4,647,447. The radionuclides permit the imaging of tissues by gamma scintillation photometry, positron emission tomography, single photon emission computed

isolated directly from cells that have been transformed with expression vectors, described herein in more detail. The invention hybrid/chimeric transactivator (fusion) polypeptide, biologically active fragments, and functional equivalents thereof can also be produced by chemical synthesis. As used herein, "biologically active fragment" refers to any portion of the polypeptide that can assemble into an active protein. Synthetic polypeptides can be produced using Applied Biosystems, Inc. Model 430A or 431A automatic peptide synthesizer (Foster City, CA) employing the chemistry provided by the manufacturer.

Modification of the invention nucleic acids, polynucleotides, polypeptides, peptides or proteins with the following phrases: "recombinantly expressed/produced", "isolated", or "substantially purc", encompasses nucleic acids, polynucleotides, polypeptides, peptides or proteins that have been produced in such form by the hand of man, and are thus separated from their native *in vivo* cellular environment. As a result of this human intervention, the recombinant nucleic acids, polynucleotides, polypeptides, peptides and proteins of the invention are useful in ways that the corresponding naturally occurring molecules are not, such as identification of selective drugs or compounds.

The present invention provides for non-human transgenic animals carrying transgenes encoding chimeric transactivator proteins. These transgenic animals may further comprise a gene of interest under the control of hypoxia responsive elements (HREs). In various embodiments of the invention, the transactivator protein may constitutively enhance the expression of the gene of interest. Alternatively, the transactivator protein may only enhance the expression of the gene of interest under certain conditions; for example, and not by way of limitation, by induction. The recombinant DNA molecules of the invention may be introduced into the genome of non-human animals using any method for generating transgenic animals known in the art.

1989; Simons et al., *Bio/Technology* 6:179-183, 1988; Swanson et al., *Bio/Technology* 10:557-559, 1992; Velander et al., *Proc. Natl. Acad. Sci., USA* 89:12003-12007, 1992; Hammer et al., *Nature* 315:680-683, 1985; Krimpenfort et al., *Bio/Technology* 9:844-847, 1991; Ebert et al., *Bio/Technology* 9:835-838, 1991; Simons et al., *Nature* 328:530-532, 1987; Pittius et al., *Proc. Natl. Acad. Sci., USA* 85:5874-5878, 1988; Greenberg et al., *Proc. Natl. Acad. Sci., USA* 88:8327-8331, 1991; Whitelaw et al., *Transg. Res.* 1:3-13, 1991; Gordon et al., *Bio/Technology* 5:1183-1187, 1987; Grosveld et al., *Cell* 51:975-985, 1987; Brinster et al., *Proc. Natl. Acad. Sci., USA* 88:478-482, 1991; Brinster et al., *Proc. Natl. Acad. Sci., USA* 85:836-840, 1988; Brinster et al., *Proc. Natl. Acad. Sci., USA* 82:4438-4442, 1985; Al-Shawi et al., *Mol. Cell. Biol.* 10(3):1192-1198, 1990; Van Der Putten et al., *Proc. Natl. Acad. Sci., USA* 82:6148-6152, 1985; Thompson et al., *Cell* 56:313-321, 1989; Gordon et al., *Science* 214:1244-1246, 1981; and Hogan et al., *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratory, 1986).

Another technique, homologous recombination of mutant or normal versions of these genes with the native gene locus in transgenic animals, may be used to alter the regulation of expression or the structure of the invention polypeptides (see, Capecchi et al., *Science* 244:1288, (1989); Zimmer et al., *Nature* 338:150, (1989)). Homologous recombination techniques are well known in the art. Homologous recombination replaces the native (endogenous) gene with a recombinant or mutated gene to produce an animal that cannot express native (endogenous) protein but can express, for example, a mutated protein which results in altered expression of invention fusion polypeptides.

In contrast to homologous recombination, microinjection adds genes to the host genome, without removing host genes. Microinjection can produce a transgenic animal that is capable of expressing both endogenous and

The present invention further provides a method for providing biologically active chimeric human-viral transactivator protein to the cells of an individual with a hypoxia-associated disorder comprising introducing into a such individual an amount of invention vectors effective to infect and sustain expression of biologically active chimeric human-viral transactivator protein in cells in which the associated transcription factor is absent, insufficient or nonfunctional therein. Invention vectors may be delivered to the target cells as a pharmaceutical composition comprising the vector and a pharmaceutically acceptable carrier. The vector may be delivered to target cells by methods known in the art, for example, intravenous, intramuscular, intranasal, subcutaneous, intubation, lavage, and the like.

Accordingly, the present invention provides alternative approaches in which the expression of a range of potentially beneficial genes is induced following the expression of biologically active mammalian transcription factors.

HIF-1α was cloned by PCR from HeLa cell cDNA and inserted into the expression vector, pcDNA3 (Clontech, Palo Alto, CA; Invitrogen, San Diego, CA). In this plasmid, expression of the gene is controlled by the CMV promoter. Following confirmation of the structure of the construct by sequencing, it was tested in HeLa and 293 cells by cotransfection with reporter plasmids in which transcription of the luciferase gene is controlled by either the EPO promoter/enhancer or the VEGF promoter. Induction of HIF-1α activity was accomplished by treatment of the cells with wither $CoCl_2$ or desferrioxamine, both of which are known to induce HIF-1α activity by a mechanism similar to hypoxia. This assay confirmed that the HIF-1α protein was indeed active.

Two HIF-1α/VP16 hybrids were constructed, the first was truncated

Recombinant plasmids

The full-length (aal-826) HIF-1α gene was isolated by PCR (Advantage cDNA PCR Kit, Clontech, Palo Alto, CA) from a HeLa cell cDNA library (Clontech) using the primers set forth in SEQ ID NO's 1 and 2 and inserted between the KpnI and XbaI sites of the expression vector, pcDNA3 (Invitrogen, Carlsbad, CA). In this plasmid, gene expression is controlled by the cytomegalovirus (CMV) immediate early enhancer/promoter. The HIF-1α/VP-16 hybrid was constructed by truncating HIF-1α at aa390 (an Afl2 site) and then joining the transactivation domain of HSV VP-16 downstream. A VP16 fragment (aa 413-490) with Afl2 and XbaI ends was amplified by PCR using Vent polymerase (New England Biolabs, Beverly, MA) and the primers set forth in SEQ ID NO's 3 and 4 and this fragment was cloned into the appropriate sites of the pcDNA3/HIF-1α construct. A related construct (pcDNA3/HIF/VP-16/R1) was produced by truncating HIF-1α at aa530 by partial digestion with EcoR1 (Figure 2). The integrity of all sequences generated by PCR was verified by DNA sequencing using an Applied Biosystems 377 DNA Sequencer. All cloning manipulations were carried out following standard procedures (Sambrook, J. et al., *Molecular Cloning, A Laboratory Manual 2d Ed.* (Cold Spring Harbor, NY, 1989)). Restriction enzymes and DNA-modifying enzymes were obtained from ether New England Biolabs or Life Technologies, Inc. (Gaithersburg, MD)and used according to the maunfacturer's specifications. Plasmid DNAs were purified with kits obtained from Qiagen (Chatsworth, CA). The plasmid construct expressing human $VEGF_{165}$ ($phVEGF_{165}$) has been described previously (Tsurumi, et al., *Circulation* 96:II-382-II-388 (1997)). Luciferase reporter plasmids (EPO-luc and VEGF-luc) were generously provided by Dr. H. Franklin Bunn (Brigham and Women's Hospital, Harvard Medical School).

"HIF-4/VP-16.AflII" and "HIF-4/VP-16.R1", respectively.)

Prior to transfection, cells were plated in 6 cm dishes at a density of 1×10^6 (239) or 2.5×10^5 (HeLa) cells per dish. Cells were co-transfected with the HIF-1α or HIF-1α/VP16 expression plasmids and a luciferase reporter plasmid containing either the EPO enhancer/promoter or the VEGF promoter.

Transfection was carried out by calcium phosphate precipitation using the ProFection™ kit (Promega, Madison, WI) using 5μg each of the HIF-1α/VP16 expressing constructs and the reporter plasmid per dish. At 24 hours post-transfection, cells were induced by adding fresh media containing either 100μg/ml $CoCl_2$ or 100μg/ml desferrioxamine. At 16 hours post-induction, the cells were harvested and the luciferase activity was measured using a luciferase assay kit (Promega) according to the manufacturer's instructions.

Induction of HIF-1 activity was simulated by treatment with desferrioxamine, an iron chelator, which acts via a mechanism similar to hypoxia. As was predicted for the HIF-1α/VP-16 hybrid transcription factor, luciferase expression was constitutive and not dependent of desferrioxamine induction. Luciferase expression in uninduced and desferrioxamine-treated cells was not significantly different. In contrast, luciferase activity in cells transfected with the full-length HIF-1α construct was increased 4-5 fold following treatment with desferrioxamine. Furthermore, transcriptional activation achieved by the HIF-1α/VP16 hybrid in uninduced cells was 10-20-fold higher than that observed in desferrioxamine-treated cells transfected with the full-length HIFα. Overall, levels of luciferase expression activated by HIF-1α were not much higher than the background levels attributable to endogenous HIF-1 activity in the HeLa cells.

As shown in Figure 6, subsequent studies have confirmed these findings. Figure 6, left, shows luciferase activity produced using an EPO 3' enhancer-

analyses have confirmed an apparent difference in activity between the two constructs. Specifically, the HIF-1α/VP16/Afl2 construct (truncated at amino acid 390 of HIF-1α) is more active than the longer HIF-1α/VP16/R1 construct, both with respect to activation of transcription of luciferase reporter constructs and up-regulation of endogenous VEGF and EPO gene expression in HeLa and Hep3B cells. This difference in activity may be due to the presence in the longer construct of a portion of an "oxygen-dependent domain" of HIF-1α which is reported to confer instability under normoxic conditions, (see Huang et al., *PNAS* 95:7987-7992 (1998)).

In additional analyses of VEGF production, HeLa cells (3×10^5 cells/60 mM dish) were transfected with Lipofectamine (Life Technologies, Inc; 3.7 μg DNA, 14 μl of Lipofectamine) in Opti-MEM media (Life Technologies, Inc.) for 17 hr. Seven hours later, one set of dishes was treated with 100 μM desferrioxamine. At 42 hr post-induction, the culture medium was harvested and the cells were lysed in 250 μl lysis buffer (0.5% NP-40, 1 mM EDTA, 50 mM Tris (pH 8.0), 120 mM NaCl, 100 μM PMSF, 0.1 U/ml Aprotinin, 1 μM Pefabloc, 5 μg/ml Leupeptin). VEGF concentration was assayed as above and the total cell protein was analyzed using the Bio-Rad (Hercules, CA) protein assay. ELISA values were normalized to total cell protein.

Rat C6 glioma cells (American Type Culture Collection, Rockville MD) were seeded onto 12-well culture plates 3 days before transfection and cultured with DME (Life Technologies, Inc) supplemented with 10% FBS (Sigma, St. Louis, MO). Transfection was performed essentially as described previously (Lee, E.R. *et al. Hum. Gene Ther.* 7:1701-1717 (1996)). Briefly, plasmid DNA was complexed with an equal volume of cationic lipid GL#67 to obtain a final concentration of 80:20 μM (DNA:lipid). Lipid/DNA complexes

human genes. This element is located within a 28 bp region that is identical in the rat and human VEGF genes.

Example 4: Analysis of the HIF-1α/VP16 Hybrid Transcription Factor in the Rabbit Hindlimb Ischemia model:

A. Serum VEGF Level

Naked plasmid DNA encoding either the HIF-1α/VP16 hybrid gene (pHIF-1α/VP16) or human $VEGF_{165}$ ($phVEGF_{165}$) was administered by injection into the medial large, adductor and semimembranous muscles of rabbits in which the femoral artery had been excised to induce hindlimb ischemia. Serum VEGF levels were assayed by an ELISA assay prepared against human VEGF. Accordingly, this assay as applied to the HIF-1α/VP16-treated group may not be quantitative as the endogenous rabbit protein is produced in these animals. However, the kinetics and persistance of VEGF expression may be compared among the groups. Before treatment, serum VEGF levels were almost undetectable and similar for each group. However, at 3 days post-administration, the VEGF levels increased to 10.5 (±3.9) pg/ml in the $phVEGF_{165}$-transfected animals and to 26.3 (±4.6) pg/ml in the pHIF-1α/VP16-treated group. The VEGF levels in the pHIF-1α/VP16-treated animals were still high, yet reduced (14.9 ± 3.0 pg/ml) at 5 days after treatment. In contrast, at 5 days, serum VEGF was undetectable in the animals treated with $phVEGF_{165}$. VEGF was not detected in the control (pCMVβ-treated) group either before or at 3 and 5 days after treatment. Though VEGF protein levels had decreased by five days, expression of both transgenes, analyzed by RT-PCR, persisted to 14 days post-administration.

resting flow was similar between pHIF-1α/VP16 and phVEGF$_{165}$-treated rabbits at day 40; however, the maximal flow was significantly higher ($p<0.05$) in the animals transfected with pHIF-1α/VP16 than in the phVEGF$_{165}$-treated group. Resting and maximal blood flow in the nonischemic limb were similar among the 3 groups at day 10 as well as day 40.

E. Effect of HIF-1α/VP16 and VEGF$_{165}$ on Collateral Vessel Development

Quantitative analysis of angiographically visible collateral vessels on the medial thigh was performed by determining vascular density. At baseline (day 10) before treatment, there was no significant difference in angiographic score among the phVEGF$_{165}$, pHIF-1α/VP16 and control groups (0.38±0.03, 0.42±0.01, 0.41±0.02, respectively; P=NS). By day 40, the angiographic scores in the pHIF-1α/VP16-treated (0.61±0.01) and in the phVEGF$_{165}$-treated (0.58±0.03) rabbits were significantly higher than that of the control group (0.51±0.05). There was no statistically significant difference in angiographic score at 40 days between pHIF-1α/VP16-treated and phVEGF$_{165}$-treated groups. The principal finding accounting for the increase in angiographic score observed in the animals that received pHIF-1α/VP16 and phVEGF$_{165}$ was enhancement in so-called mid-zone collateral vessels.

To further evaluate the effect of intramuscular HIF-1α/VP16 and VEGF gene therapy upon revascularization of the ischemic hindlimb, the medial thigh muscles of the ischemic limbs were histologically examined at day 40. Capillary densities observed in the muscles of the pHIF-1α/VP16-treated group (255±13/mm^2) and phVEGF$_{165}$-treated group (210±10/mm^2) were significantly higher than that of the control group (150±4/mm^2). In addition, the capillary density was higher ($p<0.05$) in the animals transfected with pHIFα/VP16 than in the phVEGF$_{165}$-treated animals. Moreover, the capillary/muscle fiber ratios of the pHIF-1α/VP16 and phVEGF$_{165}$-transfected

F. Procedures

Intramuscular Gene Transfer

Twenty-nine rabbits were used to study the effect of intramuscular gene therapy on hindlimb ischemia. All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zeland White rabbits (4.0 to 4.3 kg) (Pine Acre Rabbitry, Norton, MA) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) after premedication with xylazine (2 mg/kg). The surgical procedures have been previously described (Takeshita, S. et al., *Am. J. Physiol.* 93:662-670 (1994a)). Briefly, the femoral artery was completely excised from its proximal origin as a branch of the external iliac artery to the point distally where it bifurcates into the saphenous and popliteal arteries. All animals were closely monitored following surgery. Analgesia (0.25 mg/kg levophanol tartrate; Hoffmann-La Roche Inc., Nutley, NJ) was administered subcutaneously for one day. Prophylactic antibiotics (enrofloxacin, Bayer Corporation, Shawnee Mission, KA) were also administered subcutaneously for a total of 5 days postoperatively.

An interval of 10 days was allowed for postoperative recovery , after which the rabbits were returned to the catheterization laboratory. Following completion of baseline measurements, four different sites in three major thigh muscles received direct injections with plasmid DNA or vehicle only (normal saline) with the use of a 3-ml syringe and a 25-gauge needle advanced through a small skin incision. For each injection, the tip of the needle was inserted typically to a depth of 3 to 5 mm in the medial large (two sites), adductor (one site), and semimembranous (one site) muscle. The detailed procedure for the muscle injection has been previously described (Tsurumi et al., *Circulation* 94:3281-3290 (1996)). This technique was used to administer 500 μg of pHIF-

(1994a)). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to that of the normal limb.

***In Vivo* Doppler Flow Measurement**

Blood flow was quantified *in vivo* before selective internal iliac angiography on days 10 and 40 with a 0.018-in Doppler guide wire (Cardiometrics, Inc., Mountain View, CA) as previously described (Bauters, C. et al., *Am J Physiol.* 267:H1263-H1271 (1994)). The wire tip was positioned at the origin of the common iliac artery to the proximal segment of the internal iliac artery supplying the ischemic limb. Time average of the spectral peak velocity (APV) was recorded at rest and maximal APV was recorded after bolus injection of 2 mg of papaverine (Sigma, St. Louis, MO).

Doppler-derived blood flow (Q_D) was calculated as $Q_D = (\pi d^2/4)(0.5 \times APV)$, where d is vessel diameter, and APV is time average of the spectral peak velocity. The luminal diameter of the iliac artery was determined angiographically with an automated edge-detection system that has been validated previously *in vivo*. The vascular diameter was measured at the site of the Doppler sample volume (5 mm distal to the wire tip). Cross-sectional area was calculated assuming a circular lumen. The mean velocity was estimated as 0.5 x APV by assuming a time-averaged parabolic velocity profile across the vessel. The Doppler-derived flow calculated in this fashion has been shown to correlate with flow measurements determined by electromagnetic flowmeters both *in vitro* and *in vivo* (Tsurumi et al., *Circulation* 94:3281-3290 (1996)). Because 2 mg of papaverine had no effect on vessel diameter (Ku, D.D., et al., *Am. J. Physiol.* 265:H586-H592 (1993)), the diameter measurements were used to calculate both rest and maximum flow.

Selective Angiography

Selective internal iliac angiography was performed as previously

method to detect capillary endothelial cells as previously described (Ziada, A.M., et al., *Cardiovasc. Res.* 18:724-732 (1984)) and then were counterstained with eosin. A total of 20 different fields from the two muscles were randomly selected, and the number of capillaries and myofibers counted under a 20X objective. The capillary density (capillaries/mm^2) and the capillary-to-myocyte ration were then determined.

Example 5: HIF-1α/VP16 recombinant adenoviruses

As an alternative to naked DNA, adenoviral vectors were constructed carrying the HIF-1α-based genetic construct, as described below.

Two HIF-1α/VP16 hybrids were cloned into a previral plasmid vector (CMV promotor, SV40 pA, p1X-; called "empty vector" (EV) - obtained originally from R. Doll). As is described above, these two hybrid proteins contain the DNA-binding and dimerization domains from HIF-1α (the first is truncated at aa390 of HIF-1α at an AF111 site; the second is truncated at aa 530 at EcoR1 site) and the transactivation domain from Herpes Simplex Virus VP-16 protein.

Shuttle vector EV/HIF-1α/VP-16.Af111 was linearized with BstBI and shuttle vector EV/HIF-1α/VP16.R1 was linearized with BamHI. 15μg of DNA of each vector was digested for 4 hours, then purified twice by phenol/chloroform extraction and ethanol precipitation. Pellets were resuspended in 50 μl of 0.1xTE and used for transfection (2μl of each digested DNA were run on a gel prior to transfection).

Preparation of viral backbone DNA

5μg of Ad2CMVBgal-6 DNA were digested with PshAI and SnaBI at 37°C for about 24 hours, and 1/10 of the reaction was run on 0.8% Agarose/TBE gel to check the completion of digestion. The rest of the reaction

in a 1.5 ml eppendorf tube and mix gentle. Leave mixture at room temperature for 15-30 minutes.

3. Precipitate DNA-$CaCl_2$ with 300µl of 2x HBS and incubate at RT for 0.5 to 2 hours. Then add precipitated DNA to cells and incubate for 16-17 hours.

Day Three:

Change Medium.

Day Five:

Split cells from 60 mm dish to 100mm dish.

Day Seven:

Split cells from 100mm dish to 150mm dish.

Day Nine:

Pick up 5 plagues of each transfection.

Day Eleven:

Pick up 12 more plagues from EV+RI and 10 more plagues from EV+Af111. The plagues in 250µl of growth medium were frozen and thawed 3 times and stored at -20°C.

Initial Screenings of recombinant adenoviruses

1. Infection

Day One:

Plate cells on 24 well plates at $1.4x10^5$ cells/well.

Day Two:

Infect cells (about 40-50% confluent) with 200µl of each plague at

sequences contained within the HIF-1α/VP16 adenoviral vectors is shown in Figure 7. This vector is identical to the Ad2CMVBgal-6 backbone except that the B-gal gene has been replaced with the HIF-1α/VP16 hybrid construct.

Example 6: HIF-1α/NFκB Hybrid Transactivators

Using techniques similar to those described in Example 1, we constructed a nucleic acid sequence encoding a chimeric transactivator protein comprising a DNA binding and dimerization domain from HIF-1α and a transactivation domain from NF-κB. Specifically, a DNA fragment coding for the activation domain of the p65 subunit of NFκB (Schmitz, M.L. and Baeuerle, P.A., 1991 EMBO J. 10:3805-3817, Schmitz, M.L. et al., 1994 J.Biol.Chem. 269:25613-25620, Schmitz, M.L. et al., 1995, J. Biol. Chem. 270:15576-15584) was generated by PCR amplification of the DNA sequence (Advantage cDNA PCR kit, Clontech) from a HeLa cell cDNA library (Clontech). The DNA fragment was then inserted between the Afl2 and XbaI sites of the pcDNA3/HIF-1α expression vector. This construct (pHIF-1α/NF-κB) therefore consists of aa 1-390 of HIF-1α and aa 407-551 of the NF-κB p65 subunit. The integrity of sequences generated by PCR was confirmed by DNA sequencing.

Initial *in vitro* experiments were performed to evaluate the ability of the HIF-1α/NF-κB hybrid to activate expression of the endogenous VEGF gene in HeLa cells. The pHIF-1α/NF-κB construct was transfected into duplicate plates of HeLa cells in parallel with the pHIF-1α/VP16 construct and pHIF-1α (full-length, wild-type HIF-1α gene) as a control, using methods similar to those described in Example 3. Twenty-four hours after transfection, one set of plates were exposed to desferrioxamine for induction of HIF-1α activity, the other set was left untreated. The media was harvested 48 hr after induction for

REFERENCES

Banai, S. et al., *Cardiovascular Res.* 28:1176-1179 (1994).

Blanchard, K.L. et al., *Molecular and Cellular Biology* 12(12): 5373-5385 (1992).

Blau, J. et al., *Molecular and Cellular Biology* 16(5):2044-2055 (1996).

Bunn, H.F. et al., *Physiological Rev.* 76(3):839-885 (1996).

Cress, D.W. et al., *Science* 251:87-90 (1991).

Croston, G.E. et al., *Genes & Development* 6:2270-2281 (1992).

Ema, M. et al., *Proc. Natl. Acad. Sci., USA* 94:4273-4278 (1997).

Feldman, L.J. et al., *Cardiovascular Res.* 32:194-207 (1996).

Finkel, T. et al., *FASEB J.* 9:843-851 (1995).

Forsythe, J.A. et al., *Molecular and Cellular Biology* 16(9): 4604-4613 (1996).

Guillemin, K. et al., *Cell* 89(1):9-12 (1997).

Gupta, R. et al., *Nucleic Acids Research* 24(12):2324-2330 (1996).

Hashimoto, E. et al., *Am. J. Physiol.* 267(5 Part 1):H1948-H1954 (1994).

Huang, L.E. et al., *The Journal of Biological Chemistry* 271(50): 32253-32259 (1996).

International Publication Number WO 96/39426 (12 December 1996).

International Publication Number WO 96/26742 (6 September 1996).

Jiang, B-H et al., *The Journal of Biological Chemistry* 271(30):17771-17778 (1996).

Karck, M. et al., *Ann. Thorac. Surg.* 59:699-706 (1995).

Levy, A.P. et al., *The Journal of Biological Chemistry* 270(22): 13333-13340 (1995).

Li, H. et al., *The Journal of Biological Chemistry* 271(35): 21262-21267 (1996).

Claims

1. A nucleic acid molecule encoding a biologically active chimeric transactivator protein comprising

(a) the DNA binding domain of a hypoxia inducible factor protein; and

(b) a protein domain capable of transcriptional activation.

2. The nucleic acid molecule according to claim 1, wherein the hypoxia inducible factor protein is HIF-1α.

3. The nucleic acid molecule according to claim 1, wherein the protein domain capable of transcriptional activation is derived from a protein selected from the group consisting of: HSV VP16, NFκB, a heat shock factor; p53; fos; v-jun; factor EF-C; HIV tat; HPV E2; Ad E1A; Sp1; AP1; CTF/NF1; E2F1; HAP1; HAP2; MCM1; PHO2; GAL4, GCN4, and GAL11.

4. The nucleic acid molecule according to claim 1, wherein the protein domain capable of transcriptional activation is synthetic.

5. The nucleic acid molecule according to claim 1, wherein the hypoxia inducible factor protein is HIF-1α and the protein domain capable of transcriptional activation is a transcriptional activation domain from HSV VP16.

6. The nucleic acid molecule according to claim 1, wherein the hypoxia inducible factor protein is HIF-1α and the protein domain capable of transcriptional activation is a transcriptional activation domain from NFκB.

7. The nucleic acid molecule according to claim 2, 5 or 6, wherein the DNA binding domain of HIF-1α comprises amino acids 1-390.

a cell under normoxic conditions, said method comprising the step of introducing into said cell a nucleic acid molecule according to any one of claims 1-7, operatively linked to an expression control sequence which directs its expression in said cell.

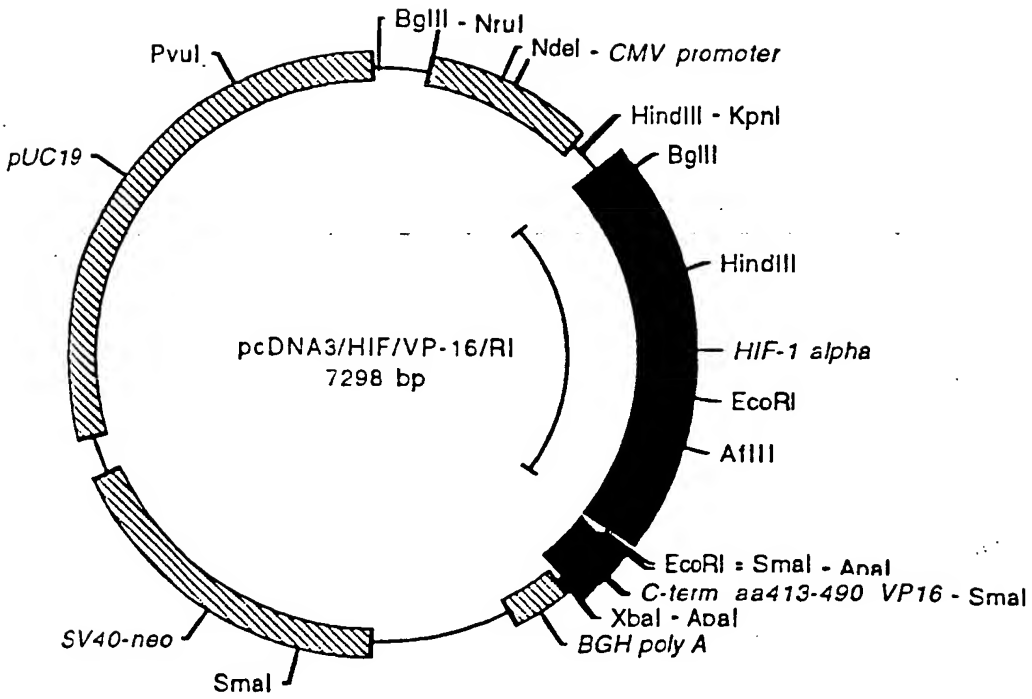
17. A method for reducing ischemic tissue damage in a subject having a hypoxia-associated disorder comprising the steps of administering to said subject an effective amount of a pharmaceutical composition according to claim 14.

18. A method for reducing ischemic tissue damage in a subject having a hypoxia-associated disorder comprising the steps of:

(a) isolating cells to be implanted into said subject

(b) introducing into said cells an expression vector according to any one of claims 8-11; and

(c) implanting said cells containing said expression vector into said subject.


BglII - NruI
NdeI - CMV promoter
HindIII - KpnI
BglII
HindIII
HIF-1 alpha
EcoRI
AflII
EcoRI = SmaI - ApaI
C-term aa413-490 VP16 - SmaI
XbaI - ApaI
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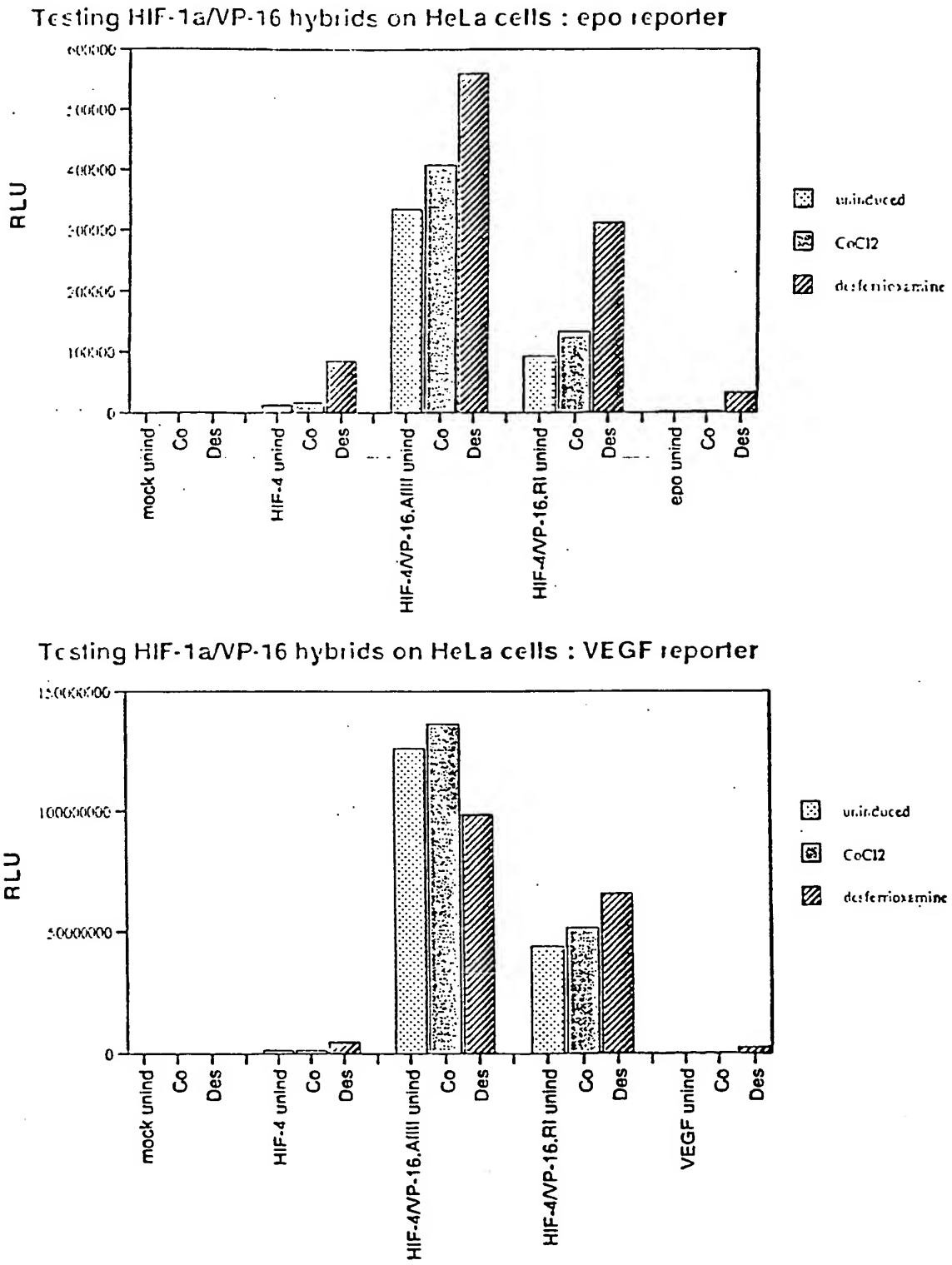
PvuI
pcDNA3/HIF/VP-16/RI
7298 bp

Figure 2


Testing HIF-1a/VP-16 hybrids on HeLa cells : epo reporter
RLU
mock unind
Co
Des
HIF-4 unind
Co
Des
HIF-4/VP-16.AIII unind
Co
Des
HIF-4/VP-16.RI unind
Co
Des
epo unind
Co
Des
uninduced
CoCl2
desferrioxamine
Testing HIF-1a/VP-16 hybrids on HeLa cells : VEGF reporter
RLU
100000000
50000000
0
mock unind
Co
Des
HIF-4 unind
Co
Des
HIF-4/VP-16.AIII unind
Co
Des
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Co
Des
VEGF unind
Co
Des
uninduced
CoCl2
desferrioxamine

Figure 4

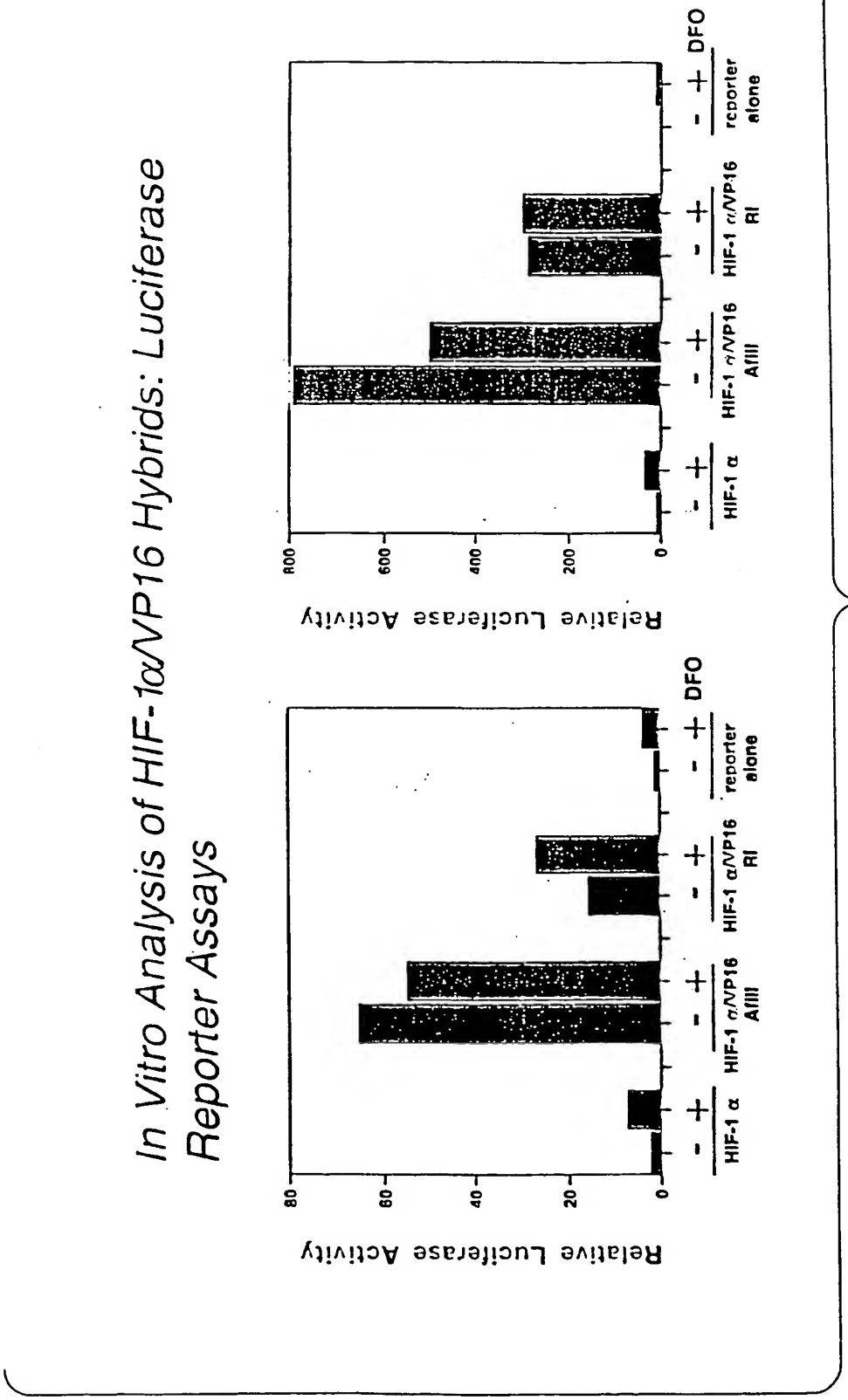
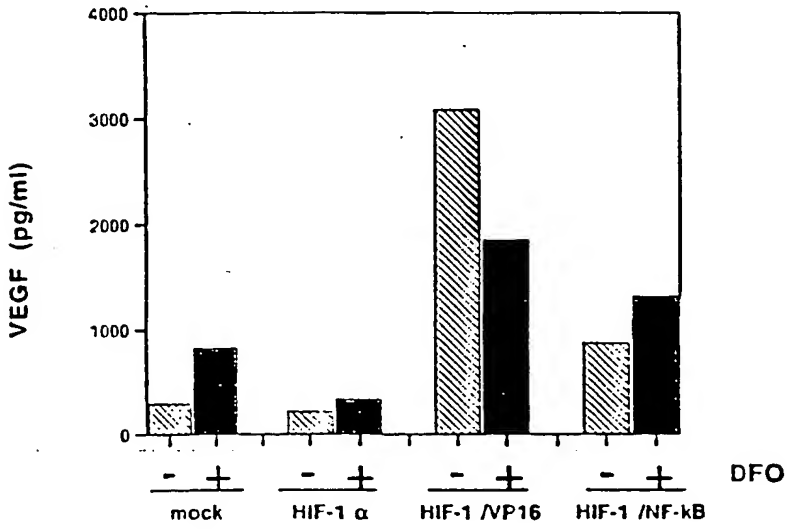

In Vitro Analysis of HIF-1α/VP16 Hybrids: Luciferase Reporter Assays
Relative Luciferase Activity
0
20
40
60
80
HIF-1 α
HIF-1 α/VP16 AIIII
HIF-1 α/VP16 RI
reporter alone
- + - + - + - + DFO
Relative Luciferase Activity
0
200
400
600
800
HIF-1 α
HIF-1 α/VP16 AIIII
HIF-1 α/VP16 RI
reporter alone
- + - + - + - + DFO

Figure 6

Comparison of HIF-1α/VP16 and HIF-1α/NFkB by Transfection in HeLa cells: VEGF levels



Comparison of HIF-1α/VP16 and HIF-1α/NFkB by Transfection in Hep3B cells: EPO levels

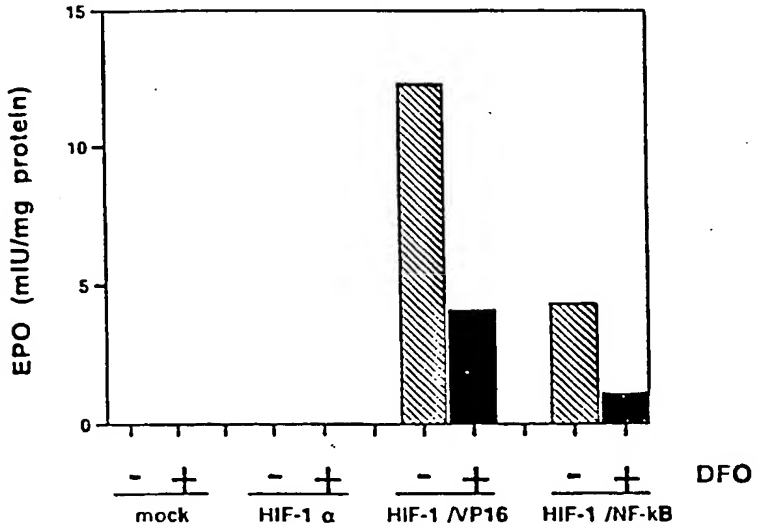


Figure 8

INTERNATIONAL SEARCH REPORT

onal Application No
PCT/US 98/25753

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/62 C12N15/86 C12N5/10 C07K14/47
A61K31/70 A61K38/17 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	K.G. SHYU ET AL.: "Naked DNA encoding a hypoxia-inducible factor 1 alpha (HIF-1 alpha)/VP16 hybrid transcription factor enhances angiogenesis in rabbit hindlimb ischemia: an alternate method for therapeutic angiogenesis utilizing a transcriptional regulatory system" CIRCULATION, vol. 98, no. 17, 27 October 1998, XP002096345 Williams & Wilkins, Baltimore, MD, US Abstract no.342 ---	1-18
A	WO 96 39426 A (UNIV JOHNS HOPKINS MED) 12 December 1996 cited in the application see the whole document --- -/--	1-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 March 1999	29/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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INTERNATIONAL SEARCH REPORT

onal Application No
PCT/US 98/25753

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	M. EMA ET AL.: "A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1-alpha regulates the VEGF expression and is potentially involved in lung and vascular development" PROC. NATL. ACAD. SCI., vol. 94, April 1997, pages 4273-4278, XP002096344 NATL. ACAD. SCI.,WASHINGTON,DC,US; cited in the application see the whole document ---	1-18
P,A	WO 98 31701 A (UNIV TEXAS) 23 July 1998 see the whole document -----	1-18

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INTERNATIONAL SEARCH REPORT

Information on patent family members

onal Application No
PCT/US 98/25753

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9639426 A	12-12-1996	AU 6332696 A	24-12-1996
		CA 2222279 A	12-12-1996
		EP 0833840 A	08-04-1998
WO 9626742 A	06-09-1996	US 5792453 A	11-08-1998
		AU 5028796 A	18-09-1996
		AU 5457096 A	31-10-1996
		CA 2188575 A	06-09-1996
		CN 1174509 A	25-02-1998
		EP 0760682 A	12-03-1997
		JP 10501423 T	10-02-1998
WO 9831701 A	23-07-1998	US 5695963 A	09-12-1997
		AU 6242098 A	07-08-1998

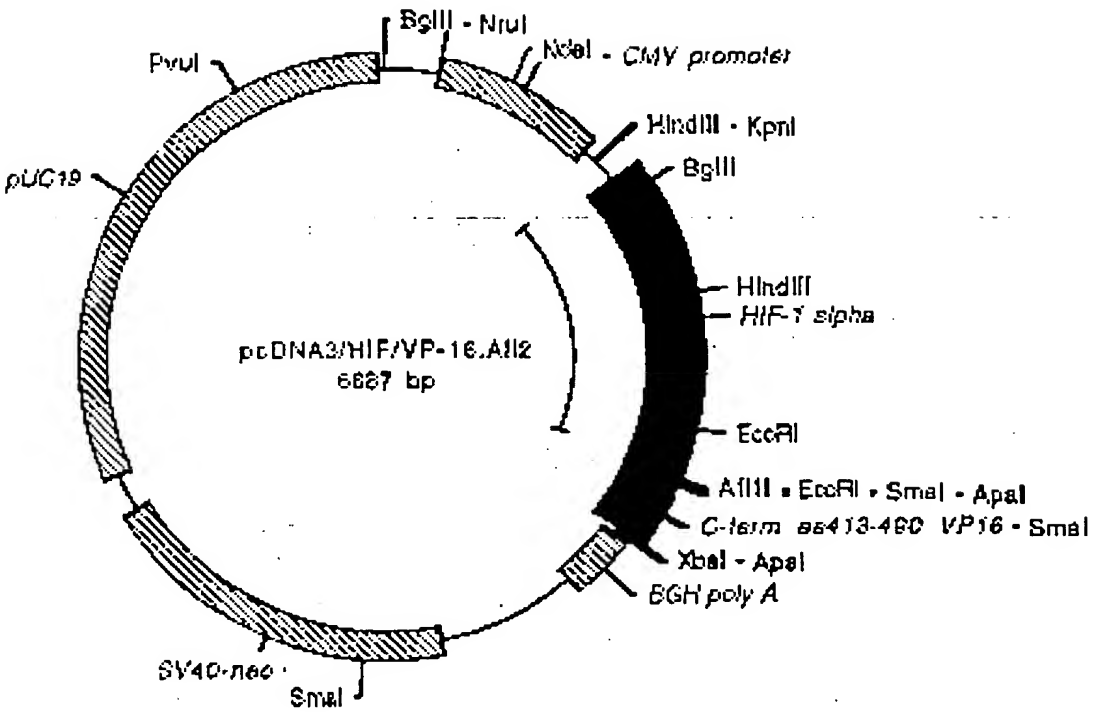
BglII - NruI
NdeI - CMV promoter
HindIII - KpnI
BglII
HindIII
HIF-1 alpha
EcoRI
AflII - EcoRI - SmaI - ApaI
C-term aa413-490 VP16 - SmaI
XbaI - ApaI
BGH poly A
SmaI
SV40-neo
pUC19
PvuI
pcDNA3/HIF/VP-16.Afl2
6687 bp

Figure 1

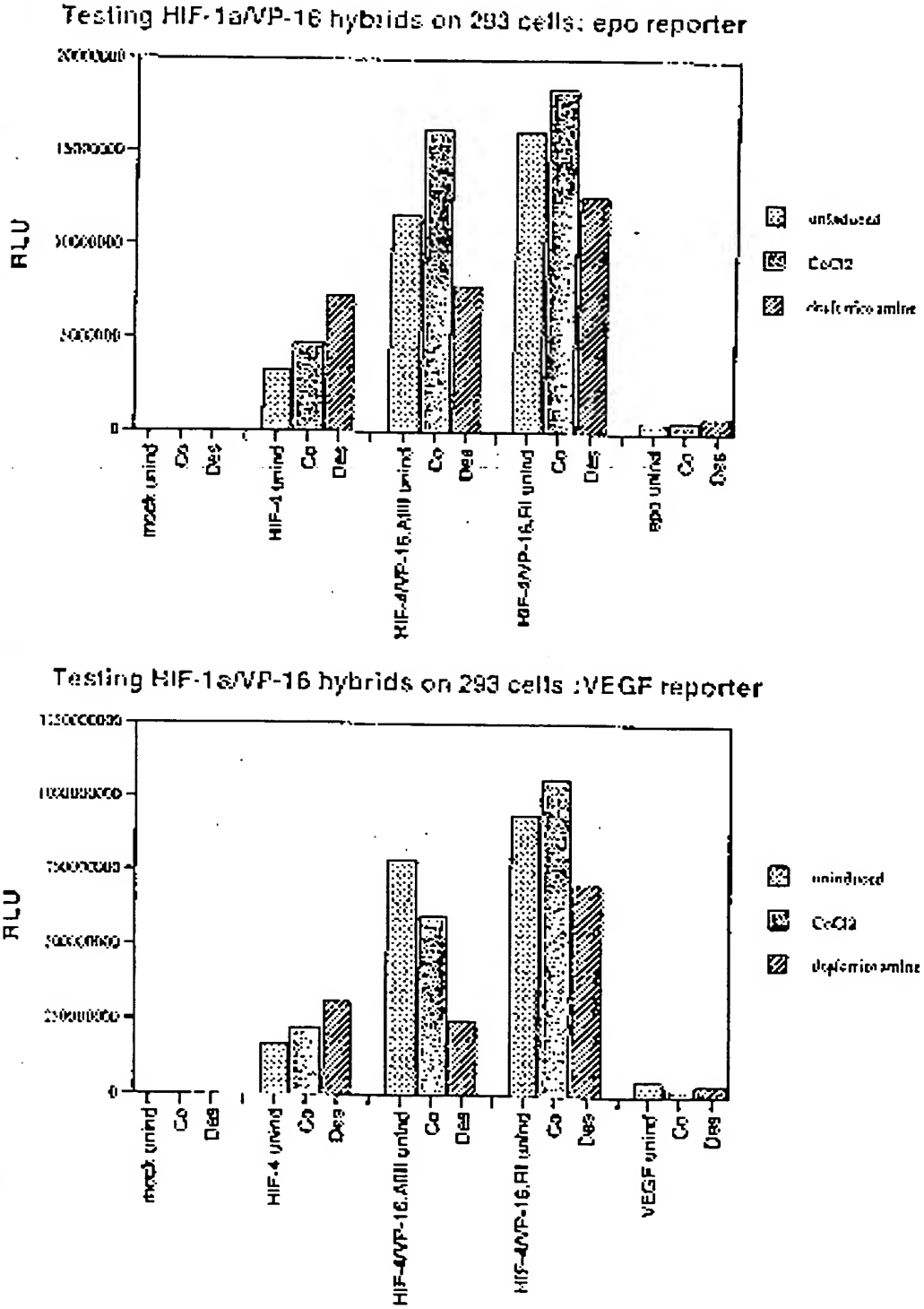
Testing HIF-1a/VP-16 hybrids on 293 cells: epo reporter
RLU
unstimulated
CoCl2
desferrioxamine
mock unind
Co
Des
HIF-4 unind
HIF-4/VP-16,AIII unind
HIF-4/VP-16,RI unind
epo unind
Testing HIF-1a/VP-16 hybrids on 293 cells :VEGF reporter
RLU
unstimulated
CoCl2
desferrioxamine
mock unind
HIF-4 unind
HIF-4/VP-16,AIII unind
HIF-4/VP-16,RI unind
VEGF unind

Figure 3

Production of VEGF in HeLa cells transfected with HIF constructs

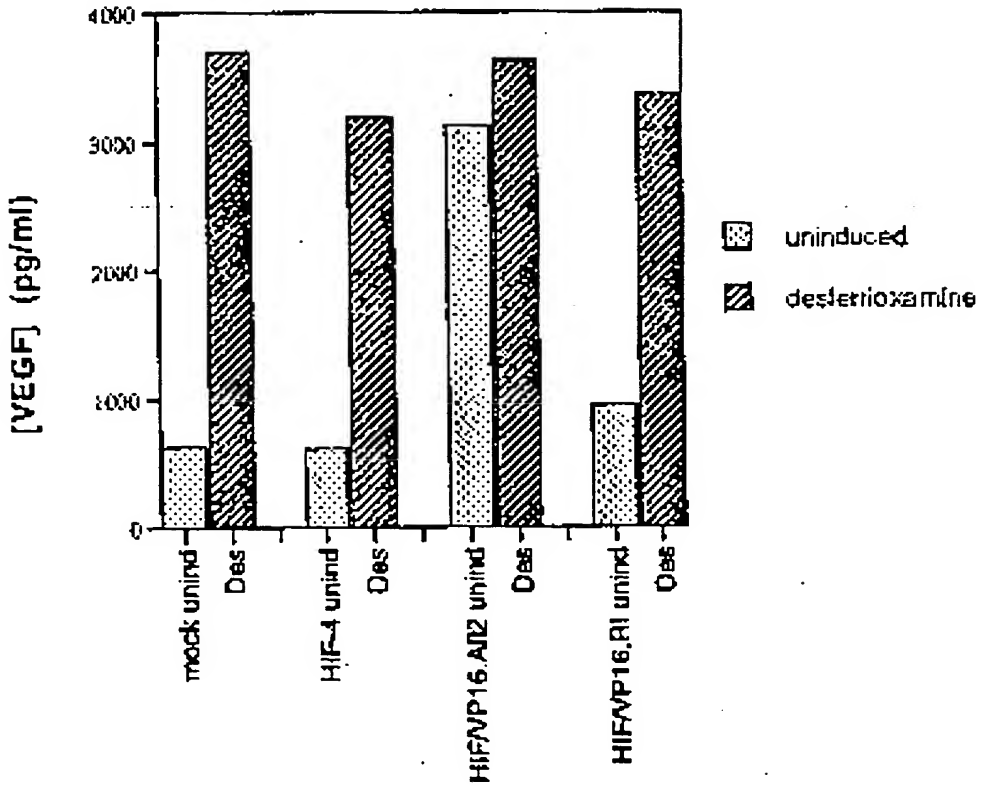

Figure 5